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Histological analysis of a Becker muscular dystrophy case, diurnal expression of dystrophin in control mice and decreased expression of dystrophin in Bmal1 knockout mice

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Running title; Sato et al: Histological analysis of a Becker muscular dystrophy and dystrophin expression

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Abbreviations: BMD: Becker muscular dystrophy; DMD: Duchenne muscular dystrophy; Bmal1: Brain and muscle Arnt-like protein 1; ZT: zeitgeber time
Key words: dystrophin, Becker muscular dystrophy, Bmal1, immunohistochemistry

Abstract
Becker muscular dystrophy (BMD) is a hereditary disease characterized by dystrophin deletion that consequently induces muscle weakness, cardiac hypertrophy and cardiac failure; These conditions are similar to those in Duchenne muscular dystrophy. The circadian rhythm is a physiological phenomenon that is predominantly regulated by the transcription and translation of clock genes. Bmal1 (Brain and muscle Arnt-like protein 1) is one of the core clock genes, and its deficiency disturbs the circadian rhythm, results in cardiac hypertrophy and cardiac failure. Dystrophin expression under diurnal conditions and in Bmal1 deficiency is yet to be elucidated. In this study, we analyzed the heart and lungs sampled during a BMD autopsy. Macroscopical examination revealed a large heart and dilated cardiomyopathy. Microscopical examination revealed an undulated structure, as well as the degeneration, and necrosis of myocardial cells. We also analyzed dystrophin expression in tissues obtained from human autopsies and mice. In human autopsy cases, dystrophin expression was lower in the heart with BMD compared that in the heart with non-BMD hypertrophy. In the heart and muscle of control mice, dystrophin expression was higher at ZT0 than at ZT12. The dystrophin expression was found to be lower in heart-specific Bmal1 knockout mice compared to that in the control mice. Hence, our study indicated that BMD was closely associated with cardiac hypertrophy and cardiac failure, while dystrophin had a diurnal expression pattern in control mice that was regulated by Bmal1.

Introduction
Dystrophin is one of the largest genes with 79 exons-located on the X chromosome (Muntoni et al., 2003). Becker muscular dystrophy (BMD) is a hereditary disease with high/low dystrophin deletion frequency (Bushby et al., 1993). The most common deletion has been observed in exons 45-47 (Bushby et al., 1993). Reduced dystrophin expressions lead to muscle weakness and damage by mechanical stress (van den Bergen et al., 2014). BMD exhibits a milder and slower clinical progression such as cardiac involvement than that by Duchenne muscular dystrophy (DMD) (Kang et al., 2016). Cardiomyopathy and ventricular dilation occur in approximately 70% of patients with
BMD, which subsequently results in heart failure (Flanigan et al., 2014; Chen et al., 2018). The American Pediatric Association recommends cardiac functional assessment in BMD and DMD (Pediatrics, 2005). Microscopic analysis has revealed that BMD patients exhibit hypertrophy and necrotic myocytes (Chun et al., 2012).

*Bmal1 (Brain and muscle Arnt-like protein 1)* is one of the clock genes, and its deficiency or abnormality leads to disturbances in circadian rhythm, insulin resistance, metabolic syndrome and cardiac failure (Nakao et al., 2018; Sato et al., 2018). In our previous study *Bmal1* exhibited circadian expression in mouse heart (Sato et al., 2017). *Bmal1* knockout (KO) mice showed increased expression levels of the inflammatory and cell cycle regulators p21, S100, CD206, and tumor necrosis factor-α (Sato et al., 2017). In addition, we demonstrated that a heart-specific *Bmal1* KO in mice increased heart size and weight, and resulted in cardiac failure (Kohsaka et al., 2014). Thus, *Bmal1* plays an important role in maintaining heart function and morphology. It has recently been reported that the loss of *Bmal1* induces muscle damage and dystrophic disease progression in DMD (Gao et al., 2020). This suggests that *Bmal1* deficiency may be associated with dystrophin regulation. In this study, we conducted immunohistochemical analysis of the BMD autopsy tissue. In addition, we examined diurnal variation in dystrophin expression in control mice and dystrophin expression in heart-specific Bmal1 KO mice.

**Material and Methods**

Tissue preparation. Histological specimens of one BMD case were retrieved from the archives of the Shingu Municipal Medical Center according to the guidelines of the Japanese Society of Pathology. This study was approved by the Wakayama Medical University Research Committee and Shingu Municipal Medical Center Ethics Committee. Clinical and histological information of a 32-year-old man revealed deletion of dystrophin exons 45-48 at the age of 6, and he was clinically diagnosed with BMD. He had severe muscular atrophy of the femur and hip. The patient had recurrent cardiac failure since ten years of age. Although he was treated with pharmacotherapy, he did not survive. His cousin was also diagnosed with BMD but the detailed clinical information was unclear. Autopsy was performed, and the heart and lungs were sampled. We prepared the heart tissue from one myocardial infarct (MI) case and
two hypertrophy cases without BMD, as previously described (Sato et al., 2017). Six-to-eight-week-old male C57BL/6 mice were housed under 12 h each of light (8AM; ZT0) and dark (8 PM; ZT12) conditions as previously described (Sato et al., 2015, 2019). We used 10 mice, and sacrificed 5 each under light and dark conditions, respectively. The mice hearts and femoral muscles were sampled at the aforementioned time points and were subjected to immunohistochemistry. Fourteen-week-old male Bmal1flx/flx and Myh6-Cre;Bmal1 flx/flx (heart-specific Bmal1 KO) mice were housed as previously described (Sato et al., 2017), and their heart tissues were sampled at ZT16. For analysis, 5 mice each were used.

Immunohistochemistry. Dystrophin expression in human and mouse tissues was evaluated using serially deparaffinized sections. Immunohistochemistry was performed using Discovery Auto-Stainer with automated protocols (Ventana Medical Systems, Inc., Tucson, AZ, USA; Roche, Mannheim, Germany), as described previously (Sato et al., 2019a,b). Dystrophin antibody (mouse monoclonal, sc-365954, 1:100) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Results
Pathological findings of the heart and lungs in BMD. The hearts and lungs of one patient with BMD were sampled via autopsy. The weights of the heart, right lung, and left lung were 590 g, 695 g, and 540 g, respectively. The heart was very large and showed dilated cardiomyopathy in macroscopic examination (Fig. 1a). Microscopic analysis revealed a marked undulated structure, degeneration and necrosis of the myocardial cells (Fig. 1b and c). Although no significant pathological findings of lungs were observed macroscopically in the lungs, focal hyperplastic alveolar epithelium and pulmonary congestion were observed on microscopic examination (Fig. 1d and e).

Dystrophin expression in the BMD heart. Dystrophin expression, when examined in BMD, MI, and hypertrophic hearts by immunohistochemistry, was found to be markedly lower in BMD than that in the membrane of myocardial cells in MI and hypertrophy (Fig. 2).
Diurnal variation in dystrophin expression in mouse heart and muscle. Subsequent immunohistochemical examination of the diurnal variation of dystrophin expression in the mouse tissues revealed higher dystrophin expression at ZT0 than that at ZT12 (Fig. 3).

Dystrophin expression in the heart of Bmal1 KO mice. Additionally, we assessed the dystrophin expression in Myh6-Cre;Bmal1^{flo/flo} heart, which revealed reduced expression in Myh6-Cre;Bmal1^{flo/flo} mice than that in control Bmal1^{flo/flo} mice (Fig. 4).

Discussion
Macroscopic examination revealed right ventricular dilation and left ventricular hypertrophy. In addition, microscopic examination revealed the undulating structure of myocardial cells, degenerated necrotic myocardial cells, and decreased dystrophin protein expression. These abnormalities may induce ventricular fibrillation and cardiac failure. These cardiac phenotypes have also been observed in BMD and DMD (Flanigan et al., 2014; Kang et al., 2016; Chen et al., 2018). Our pathological findings were compatible with BMD. The lung weight was within the normal range, and no obvious thrombus or severe inflammation was observed. Therefore, we speculate that the histological finding of hyperplastic alveolar epithelium and pulmonary congestion is a non-specific phenotype. Interestingly, dystrophin expression in both the heart and muscle was higher at ZT0 than at ZT12. We previously reported that Bmal1 expression in the heart was higher at ZT0 than at ZT12 and revealed circadian expression (Sato et al., 2017). Additionally, dystrophin expression was decreased in the heart-specific Bmal1 KO mice. These results suggest that dystrophin expression may be regulated by Bmal1 under circadian rhythm. Circadian rhythms are predominantly regulated by the transcription and translation of clock genes (Honma et al., 2002; Sato et al., 2018). Briefly, CLOCK and BMAL1 heterodimer binds to the E-boxes in the promoter of DEC, PER, and CRY, and the activated protein products of DEC, PER and CRY suppresses CLOCK and BMAL1 transactivation (Honma et al., 2002; Sato et al. 2004, 2018). The E-boxes are core elements for the regulation of the circadian rhythm (Honma et al., 2002; Sato et al., 2004, 2018). To our knowledge, dystrophin appears to
have an E-box in its promoter. We speculated that dystrophin exhibits diurnal and circadian expression through the E-box. The current study is limited and shows circadian expression of dystrophin. Further studies are needed to clarify whether CLOCK and Bmal1 induce dystrophin transcription.

Since Bmal1 KO mice showed cardiac hypertrophy and cardiac failure (Kohsaka et al., 2014), a decrease in the BMD-associated dystrophin expression may be due to Bmal1 deficiency. Further studies are needed to clarify the detailed molecular mechanism how Bmal1 deficiency decreases dystrophin expression.

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Competing interests
The authors declare no conflicts of interest.

References
suppresses epithelial cell migration and proliferation via the clock gene Dec1, which negatively regulates the expression of clock genes Dec2 and Per1. Am. J. Pathol. 189, 773-783.


**Figure legends**

Fig.1 Pathological findings of BMD. (a) Dilated cardiomyopathy was observed macroscopically in the excised surface. Arrows show dilated right ventricle. (b) Undulating structure of myocardial cells. 200 x magnification (c) Degenerated and necrotic myocardial cells. Arrow shows nuclear enlargement. 400 x magnification (d) Hyperplastic alveolar epithelium. 200 x magnification (e) Pulmonary congestion. 200 x magnification

Fig.2 Dystrophin expression decreased in heart of BMD. Dystrophin immunoreactivity in the heart of (a) BMD. 400 x magnification (b) MI. 400 x magnification (c) hypertrophy case 1. 400 x magnification (d) hypertrophy case 2. 400 x magnification.

Fig.3 Diurnal expression of dystrophin in mice heart and muscle. Dystrophin immunoreactivity in mice heart and muscle at ZT0 and at ZT12. 400 x magnification.

Fig.4 Dystrophin expression decreased in the heart of Bmal1 knockout mice. Dystrophin expression in the heart of Bmal1^{flx/flx} and Myh6-Cre;Bmal1^{flx/flx}. 400 x magnification.
Dystrophin

Bmal1\textsuperscript{flx/flx}

Myh6-Cre;

Bmal1\textsuperscript{flx/flx}